Drug Development and Industrial Pharmacy, 2012; 38(3): 365–371

ISSN 0363-9045 print/ISSN 1520-5762 online
DOI: 10.3109/03639045.2011.604331

informa
healthcare

RESEARCH ARTICLE

Physical characterization and cellular uptake of propylene glycol liposomes *in vitro*

Lu Zhang[1]*, Cui-Tao Lu[1]*, Wen-Feng Li[2]*, Jin-Guo Cheng[3], Xin-Qiao Tian[4], Ying-Zheng Zhao[1], Xing Li[1], Hai-Feng Lv[1], and Xiao-Kun Li[1]

[1]Wenzhou Medical College, Wenzhou City, Zhejiang Province, China, [2]Oncology Department, The First Affiliated Hospital of Wenzhou Medical College, Wenzhou City, Zhejiang Province, China, [3]Wenzhou Traditional Chinese Medicine Hospital, Wenzhou City, Zhejiang Province, China, and [4]Ultrasound Department, The Second Affiliated Hospital of Wenzhou Medical College, Wenzhou City, Zhejiang Province, China

Abstract

In order to facilitate the intracellular delivery of therapeutic agents, a new type of liposomes–propylene glycol liposomes (PGL) were prepared, and their cell translocation capability *in vitro* was examined. PGL was composed of hydrogenated egg yolk lecithin, cholesterol, Tween 80 and propylene glycol. With curcumin as a model drug, characterization of loaded PGL were measured including surface morphology, particle size, elasticity, encapsulation efficiency of curcumin and physical stability. Using curcumin-loaded conventional liposomes as the control, the cell uptake capacity of loaded PGL was evaluated by detection the concentration of curcumin in cytoplasm. Compared with conventional liposomes, PGL exhibited such advantages as high encapsulation efficiency (92.74% ± 3.44%), small particle size (182.4 ± 89.2 nm), high deformability (Elasticity index = 48.6) and high stability both at normal temperature (about 25°C) and low temperature at 4°C. From cell experiment *in vitro*, PGL exhibited the highest uptake of curcumin compared with that of conventional liposomes and free curcumin solution. Little toxic effect on cellular viability was observed by methyl tetrazolium assay. In conclusion, PGL might be developed as a promising intracellular delivery carrier for therapeutic agents.

Keywords: Propylene glycol liposome, intracellular delivery, curcumin, physical properties, cellular uptake

Introduction

With rapid development of new types of liposomes, researchers started to pay more attention to using different types of liposomes as targeted delivery carriers for therapeutic agents. With liposomes as drug carriers, drug toxicity can be reduced, drug stability be increased and drug release behavior be changed[1,2]. However, an ideal drug targeted delivery system is not only required to delivery drugs into tissue but also promote drugs to enter targeted cells. It is known that intracellular delivery carriers can enter the cytoplasm via endocytosis, fusion, diffusion or phospholipid exchange. And endocytosis is the major uptake pathway of most cells[3,4]. Liposome composition and particle size can affect the efficiency and pathway of cellular uptake for liposomes by influencing the interaction between particles and cells, such as adsorption and fusion of liposome onto the cell surface[5–7].

There are hydrophilic and hydrophobic regions in cell membrane which determine the transport rate of different substance from the extracellular fluid into the cytoplasm. With a fat-soluble small molecule, ethanol can affect membrane proteins and lipid membrane structure, which results in the lipid bilayer reversible disorders[8–10]. Therefore, ethanol can be used to increase

*These authors contributed equally to this work.

Address for Correspondence: Xin-Qiao Tian, Ultrasound Department, The Second Affiliated Hospital of Wenzhou Medical College, Zhejiang Province 325035, China. Tel: 86–577-86699363. E-mail: lctuua@sina.com
Ying-Zheng Zhao, Pharmacy School, Wenzhou Medical College, Zhejiang Province 325035, China. Tel: 86–577-86699363. E-mail: lctuua@yahoo.com.cn

(Received 09 May 2011; revised 14 June 2011; accepted 01 July 2011)

the permeability of cell membrane. As elastic liposomes, ethanol liposomes (ethosomes) contained 30%–45% ethanol which can provide the vesicles with soft flexible characteristics and allow them to easily penetrate into cell lipid membrane[11,12].

Though ethanol liposomes have flexible liposome shell and high penetration ability, there are few reports on using ethanol liposomes as a intracellular delivery carrier[13,14] because of the toxicity of high concentration of ethanol. Moreover, ethanol liposomes cannot maintain long-time stability based on previous investigations. With the evaporation of ethanol during storage, ethanol liposomes begin to gather which may result in particle size increase and loaded drugs' leakage.

Propylene glycol has slightly higher oil–water partition coefficient than ethanol. In our previous unreported experiments, propylene glycol has similar penetration ability as ethanol and therefore can be used as an elasticity modulator in flexible liposome formulation. With similar transform ability as ethanol liposomes, liposomes-composed propylene glycol can maintain long-time stablility during storage.

Based on our experiment, some polyols can be used as lipid membrane stabilizer. In this paper, a new type of flexible liposomes – propylene glycol liposomes (PGL), were prepared with curcumin as a model drug. With propylene glycol used as elasticity modulator and trehalose used as lipid membrane stabilizer in formulation[15], PGL may enjoy both high flexibility as ethanol liposomes and stability as lyophilized liposomes. Compared with conventional liposomes, characterization of loaded PGL were measured including surface morphology, particle size, elasticity, encapsulation efficiency of curcumin and physical stability. With Chinese hamster ovary (CHO) cells, cellular uptake assessment was observed and cytotoxicities of the carriers were evaluated.

Materials and methods

Preparation of curcumin-loaded PGL and conventional liposomes

Preparation of curcumin-loaded PGL

Curcumin-loaded PGL was prepared by ethanol injection method. Hydrogenated egg yolk phosphatidylcholine 10 mg (HEPC, Doosan Corporation Biotech BU, Kyonggi Do, Korea), cholesterol 3 mg (Chol, Beijing Chemical Reagent Corporation, Beijing, China), Tween-80 1 mg (Hubei Biological Technology Company, Hubei Province, China) and curcumin 6 mg (Hebuo Biotechnology Company, Guangzhou Province, China) were dissolved in 2 ml propylene glycol bathed in 65°C water. Then propylene glycol solution was added slowly into 10 ml 5% trehalose solution under 750 r/min rotation. After constant rotation for 30 min, PGL-containing curcumin were prepared.

Preparation of curcumin-loaded conventional liposomes

Curcumin liposomes were prepared by same ethanol injection method as curcumin-loaded PGL. HEPC 10 mg, Chol 3 mg, Tween 80 1 mg and curcumin 6 mg were dissolved in 5 ml anhydrous alcohol. Then the alcohol solution was added slowly into 12 ml 5% trehalose solution with constant rotation of 750 r/min. Alcohol in final liposome solution was removed by vacuum rotation evaporator. The vesicle suspension was driven through a microporous filter (0.6 um) by an external pressure of 2.5 bars.

Curcumin solution preparation

Curcumin solution was also prepared as blank control in following experiments. Curcumin 0.5 mg was dissolved in 1 ml dimethyl sulfoxide (DMSO) bathed in 65°C water and then curcumin solution was prepared.

Characterization of PGL and conventional liposomes

Morphology and size distribution observation

Morphology: The morphology of loaded PGL and conventional liposomes were observed by Transmission Electron Microscope (TEM) (1230, Jeol Jem Company, Tokyo, Japan) using negative staining with 1% phosphotungstic acid. The liposome sample was properly diluted with 5% trehalose solution, and a drop of the diluted sample was placed on the surface of copper grid. Then the diluted sample was stained with 1% phosphotungstic acid and dried by air. The microscopic appearance of the liposomes was examined by TEM.

Size distribution: Size distribution of loaded PGL and conventional liposomes were examined by laser particle size measuring instrument (LS800, OMEC Company, US). The average particle size of loaded PGL and conventional liposomes were reported from at least three samples.

Curcumin entrapment efficiency

Sephadex G-50 column was used to separate free curcumin from curcumin encapsulated in liposomes. Curcumin liposome solution 0.5 ml was added in a Sephadex G-50 (1.0 × 25 cm) column. The effluent was 0.02 mol/L phosphate-buffered saline (PBS) (pH7.4). The effluent liquid containing curcumin liposomes was collected and lyophilized at 5×10^{-4} Pa pressure for 20 h. Then curcumin liposomal lyophilized products were solved in anhydrous alcohol. The content of curcumin was determined by high-performance liquid chromatography (HPLC). HPLC conditions were as follows: Chromatographic column: Kromasil C_{18} (250 × 4.6 mm, 5 μm); mobile phase; acetonitrile: 4% glacial acetic acid (50:50); velocity: 1.0 mL/min; UV detected wave length: 430 nm. Each sample was conducted in triplicate. The peak at 11.701 min represented curcumin. A calibration curve was produced using commercial curcumin in the range of 5–100 μg/mL. Curcumin entrapment efficiency was calculated by the following equation[10].

$$\text{Entrapment efficiency (\%)} = \left(\frac{\text{encapsulated amount of drug}}{\text{total amount of drug}}\right) \times 100$$

Liposome elasticity

The PGL and conventional liposomes were driven through microporous filter (Pore diameter = 50 nm, Tangwei Biotecnology Company, Zhejiang Province, China), respectively, by an external pressure of 2.5 bars. Vesicle size was determined by laser particle scanner before and after filtration. The elasticities of the liposomal membranes of PGL and conventional liposomes were calculated using the following formula: $D = J \times (r_v/r_p)^2$, where D is the deformability index of the vesicle membrane, J is the amount of vesicle suspension extruded in 5 min, r_v is the size of the vesicles after extrusion and r_p is the pore size of the barrier[11].

Influence of storage temperature

To investigate the influence of storage temperature, curcumin-loaded PGL and conventional liposomes kept in 5% trehalose solution were evaluated at two different temperatures (25°C and 4°C) for 30 days. The average particle size and drug encapsulation efficiency of the vesicles were measured after 1, 2, 7, 15 and 30 days using the method described earlier.

Cellular uptake experiment in vitro

The CHOs, purchased from United States, were used for these experiment. These CHO cells were incubated in humidified room air containing 5% CO_2 at 37°C and cultured in Dulbecco's Modified Eagle Medium (DMEM) (Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (GIBCOBRL, Grand Island, NY, USA). Cells were routinely grown in 100 mm plastic tissue culture dishes (Nunc, Roskilde, Denmark) and harvested with a solution of trypsin-EDTA when they were in logarithmic phase of growth. For the cellular uptake study, CHO cells were seeded in growth medium at a density of 1×10^5 per well into a 6-well plate on coverslips.

To avoid the influence of particle size on cellular uptake, some conventional liposomes were previously extruded through 0.45 um microporous membrane to reduce the particle sizes to 289 ± 132.1 nm. After 12 h incubation, cells were treated with designed groups as follows.

Group 1 (Positive control): CHO cells treated with curcumin solution (500 μg/ml).

Group 2 (PGL): CHO cells treated with curcumin-loaded PGL containing an equivalent amount of curcumin as positive control.

Group3 (Conventional liposome 1): CHO cells treated with conventional liposome containing an equivalent amount of curcumin as positive control.

Group 4 (Conventional liposome 2): CHO cells treated with conventional liposome (extruded through 0.45 μm microporous membrane) containing an equivalent amount of curcumin as positive control.

At predetermined time point, cells were washed five times with PBS. Cell number was mounted and the fluorescence of the curcumin delivered to the cells was visualized using inverted fluorescence microscope (TS100-F, Nikon Eclipse Company, Japan). Then cell was broken with Triton X-100 for 12 h. After centrifugation with 3000 r/min for 10 min, the concentration of curcumin in supernatant of different groups was measured by HPLC.

Cytotoxicity assay

The CHO cells were seeded in growth medium at a density of 1×10^5 per well into a 96-well plate. After 12 h incubation, cells were treated with curcumin solution, PGL suspension and conventional liposome suspension. Blank control cells were treated with double-distilled water. Cells in each groups were incubated for 4 h followed by methyl tetrazolium (MTT) assay, in which blank control cells served as 100% cell viability.

Statistical analysis

All data were expressed as Mean ± SD. Statistical significance was determined by Student's *t*-test and difference was considered to be significant at $p < 0.05$.

Results and discussion

Morphology and size distribution observation

The physical characteristics of liposomes including vesicular size, polydispersity index, deformability and loading efficiency were investigated in this experiment. The morphology of PGL and conventional liposomes were also observed by TEM (Figure 1). The PGL and conventional liposomes showed some similarity in the morphology. All vesicles in PGL and conventional liposomes were found to be spherical in shape. There were some aggregation in conventional liposomes suspension, but no aggregation was found in PGL. Because propylene glycol in formulation exerted stabilizing effect to prevent vesicle aggregation, PGL maintained stable morphology longer than that of conventional liposomes.

Vesicle size, drug entrapment efficiency and deformability were reported in Table 1. PGL showed a mean particle size of 182 nm, with a polydispersity index of 0.239 (measured by laser particle scanner). The particle size of PGL was smaller than that of conventional liposomes, which might be resulted from the presence of propylene glycol in the vesicles. Propylene glycol could cause a modification of interfacial tension for vesicles and conferred PGL some degree of spatial stability, which might finally lead to a decrease in the mean vesicle size. The zeta potential also contributed to the phenomenon. PGL showed negative charges of about −13.7 mV, compared with −4.71 mV of conventional liposomes. With higher net charge, PGL could avoid vesicle accumulation and maintain long-time stability.

Curcumin entrapment efficiency

The ability of a carrier to entrap and retain drugs was very important to evaluate the potential therapeutic use of liposomes. As shown in Table 2, more than 90%

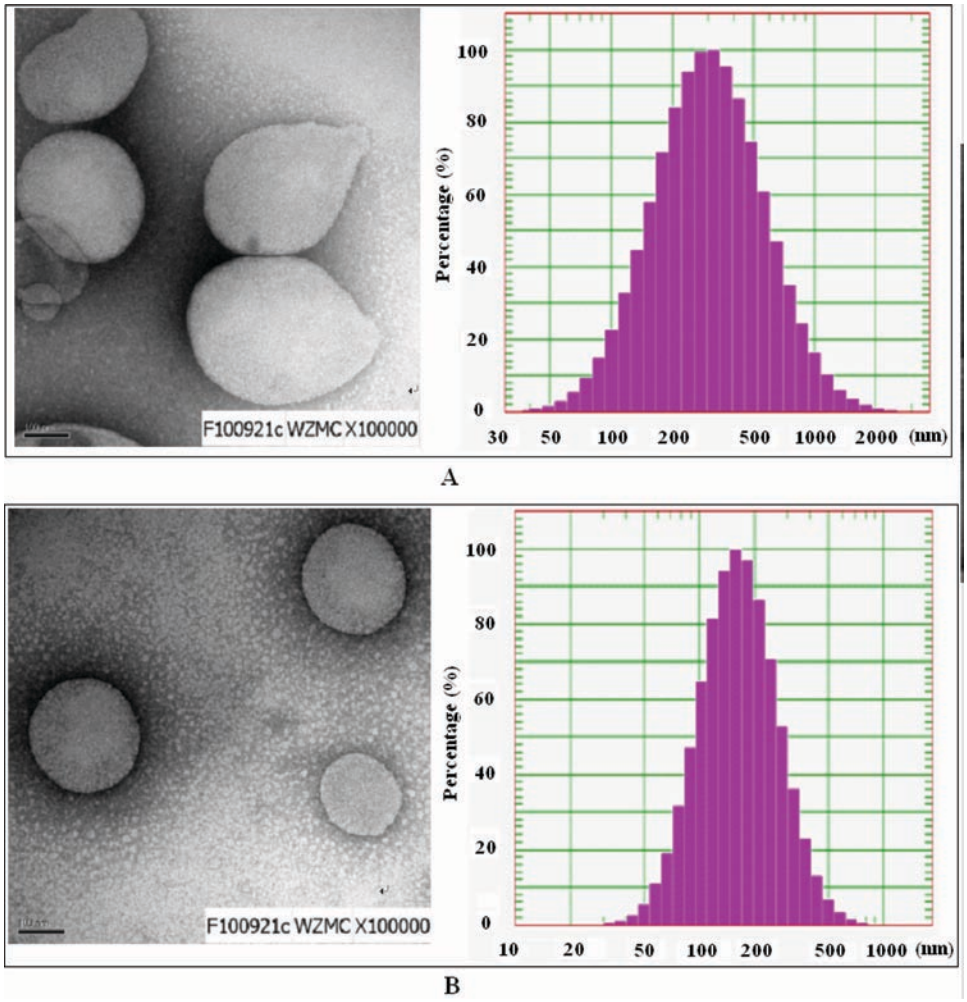

Figure 1. TEM and size distribution of conventional liposomes and PGL (A: Conventional liposomes, average diameter = 632.9 ± 484.1 nm; B: PGL, average diameter = 182.4 ± 89.2 nm).

Table 1. Particle size distribution of PGL and conventional liposomes.

Liposomal type	Size (nm)	Polydispersity index
PGL	182.4 ± 89.2	0.239
Conventional liposomes	632.9 ± 484.1	0.585

Note: Values represent Mean ± SD ($n = 3$).

Table 2. Entrapment efficiency and deformability of PGL and conventional liposomes.

Liposomal type	Curcumin entrapment efficiency (%)	Deformability
PGL	91.81 ± 5.96	48.6 ± 7.5
Conventional liposomes	70.43 ± 4.68	10.5 ± 2.5

Note: Values represent Mean ± SD ($n = 3$).

curcumin was encapsulated in PGL, while only about 70% curcumin was encapsulated in conventional liposomes. Two factors might contribute to the high entrapment efficiency of PGL. The first factor was the solubility of propylene glycol and the second might be the good affinity between propylene glycol and curcumin. With propylene glycol interpenetrating hydrocarbon chains, more space for containing curcumin could be created and high entrapment efficiency could be gained. No doubt, other factors related to propylene glycol might also enhance curcumin entrapment efficiency.

Liposomal deformability

Liposomal deformability can reflect the action of composition in vesicle membrane elasticity. As shown in Table 2, deformability of PGL (48.6 ± 7.5) was about fivefold as that of conventional liposomes (10.5 ± 2.5). From the results, the elasticity of PGL shell was improved with propylene glycol in vesicle structure. Since the interfacial tension of the PGL membrane could be reduced greatly by propylene glycol, PGL enjoyed more transformal ability than conventional liposomes[15].

Influence of storage temperature

Storage temperature has great impact on the liposomal structure. Therefore, two different storage temperature were tested in this experiment. As shown in Figure 2, there were significant differences in curcumin entrapment and particle size between PGL and conventional liposomes during 30-day storage ($p < 0.05$). Under 25°C storage, 89.7% loaded curcumin was still retained in PGL

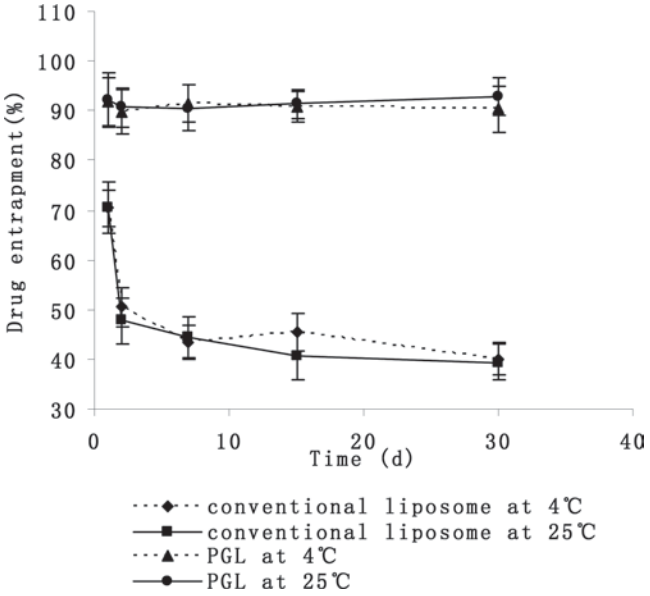

Figure 2. Curcumin entrapment (%) of conventional liposomes and PGL at normal temperature (about 25°C) and low temperature at 4°C over 30 days ($n = 3$).

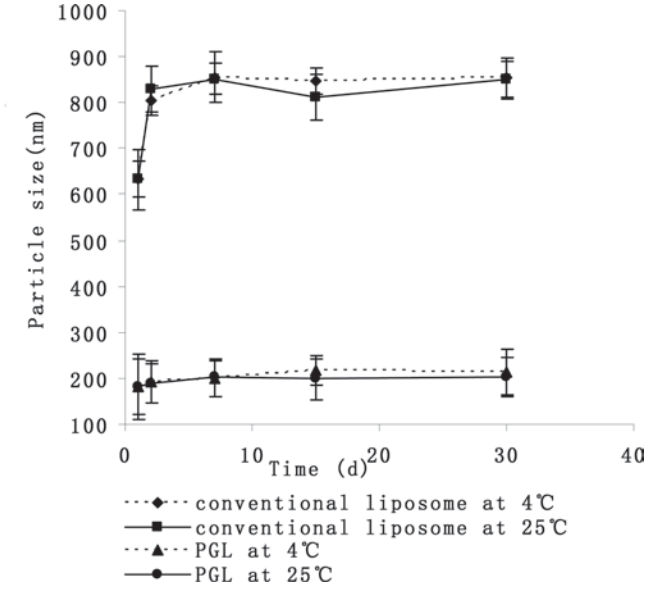

Figure 3. The average particle size (nm) of conventional liposomes and PGL at normal temperature (about 25°C) and low temperature at 4°C over 30 days ($n = 3$).

even at 30 days, compared with 38.5% curcumin retained in conventional liposomes ($p < 0.05$). Similar results were obtained under 4°C storage. Whether in PGL or conventional liposomes, curcumin entrapment at storage temperature 4°C and 25°C did not show significant difference ($p > 0.05$).

As shown in Figure 3, although little changes in particle size were found in PGL, significant changes in particle size were seen in conventional liposomes. Whether at storage temperature 4°C or 25°C, the average particle size of conventional liposomes at 30 days was significantly larger than their original size ($p < 0.05$). However, little change in particle size during 30-day storage was found

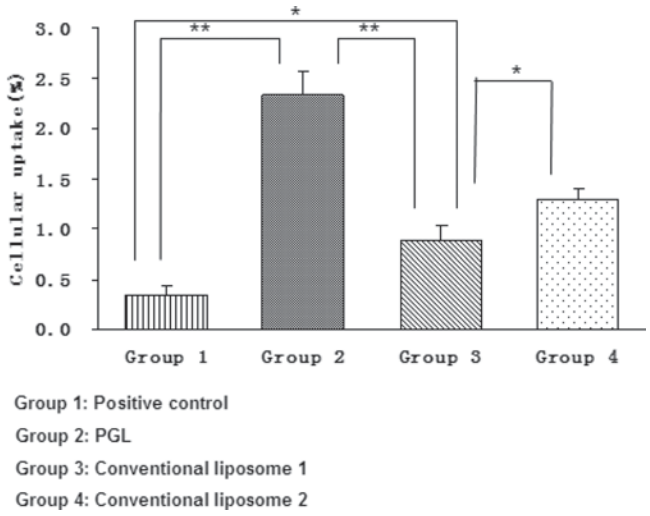

Figure 4. Cellular uptake of curcumin in CHO cells following 5 h incubation with curcumin alone, conventional liposomes, conventional liposomes extruded through 0.45 um nuclepore filter and PGL containing curcumin. Indicated values were Mean ± SD ($n = 3$). *$p < 0.05$,**$p < 0.01$.

in PGL ($p > 0.05$). This difference between PGL and conventional liposomes may be a result of storage condition and the possible occurrence of ion–charge interactions. Traditionally, liposomal interlamellar distance can be increased during long-time storage, which consequently cause an expansion of liposomal size[28].

According to the results of curcumin entrapment and particle size, loaded PGL maintained stablility during storage under normal temperature (about 25°C) or low temperature at 4°C.

Thorough mechanism about the protection effect of PGL on curcumin is not clear. However, the presence of propylene glycol in PGL might be the key factor. Propylene glycol is a good cosolvent for curcumin. With injection method preparation, curcumin could be encapsulted in PGL, because propylene glycol was in the core of PGL. With smaller size of vehicle and better stability, curcumin-loaded PGL enjoyed better protection than conventional liposomes.

Cellular uptake *in vitro*

Using curcumin solution as the control, cellular uptake of conventional liposomes and PGL were investigated in CHO cell lines. As shown in Figures 4 and 5, Group 1 (Positive control) showed minimal intracellular accumulation in the cells among test groups. For the three groups treated with loaded liposomes, the order of curcumin cellular uptake *in vitro* was: Group 2 (PGL) > Group 4 (Conventional liposome 2) > Group 3 (Conventional liposome 1). As shown in Figure 4, the differences among different groups were significant. From the order, PGL showed the highest level cell uptake and accumulation in CHO cells. Considering liposomal deformability results, PGL had better deformability and penetration ability than conventional liposomes. Therefore, PGL enjoyed better value in curcumin cellular uptake than conventional liposomes.

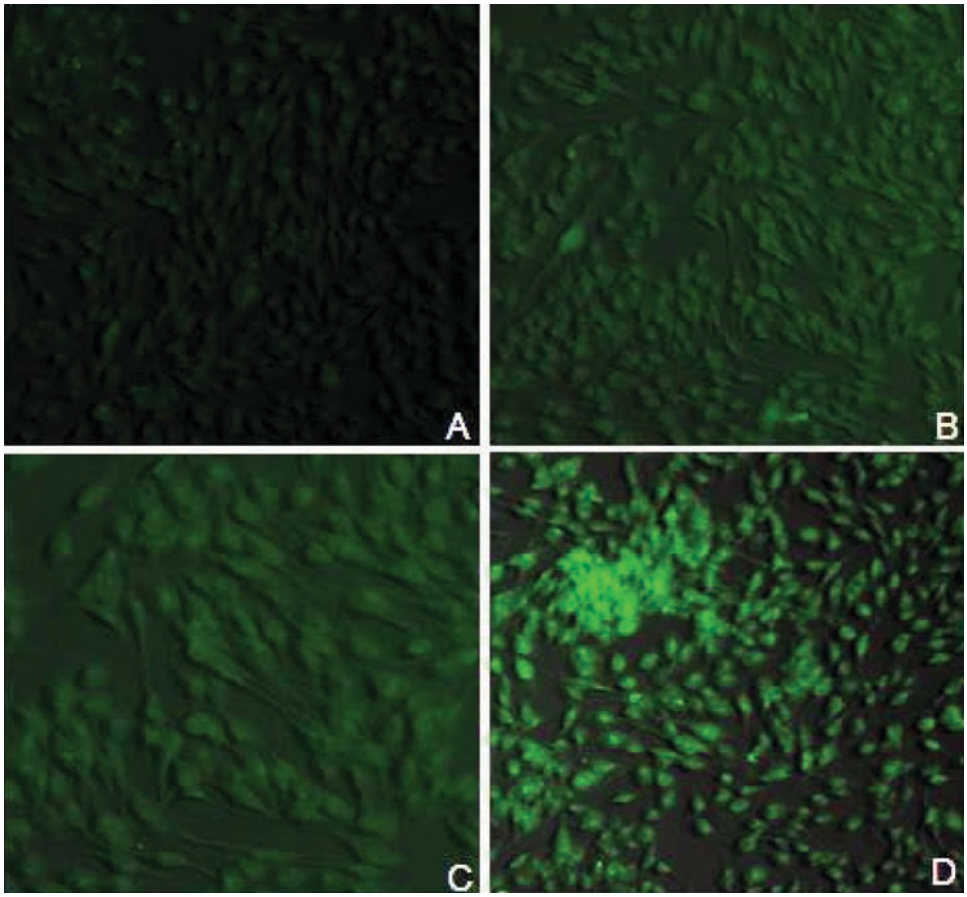

Figure 5. Fluorescence microscopy of CHO cells treated with (A) curcumin alone, (B) conventional liposome, (C) conventional liposomes extruded through 0. 45 um nuclepore filter, (D) PGL containing curcumin at 37°C for 5 h. Curcumin showed only minimal intracellular accumulation, whereas liposomal formulations increased the cellular association of curcumin. As observed in (B) and (C), PGL showed a drastic increase in cellular internalization of the curcumin.

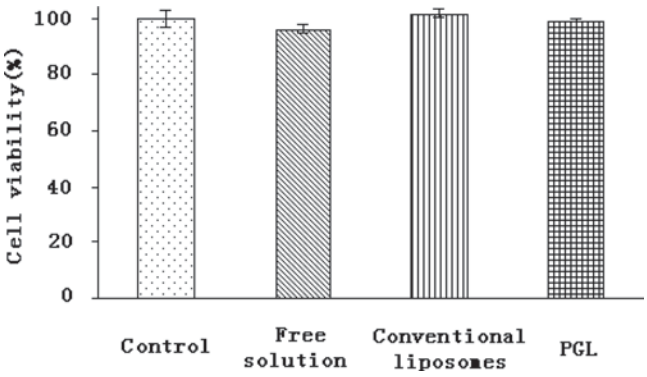

Figure 6. Cytotoxicities of empty liposomes in CHO cells by MTT assay under the same experimental conditions as in the cell uptake studies. Cells were treated with curcumin alone, conventional liposomes, PGL for 4 h. None of the formulations were cytotoxic to CHO. Data were expressed as Mean ± SD ($n = 3$).

In addition, liposome particle size might exert influence on curcumin cellular uptake. From the comparison of Group 4 (liposome particle size = 289 ± 132.1 nm) and Group 3 (liposome particle size = 632.9 ± 484.1 nm), small particle size could facilitate more amount of curcumin enter cells. This result partially confirmed the hypothesis proposed by Gao[16–18]. In this hypothesis, large liposomes needed stronger driving force and additional energy in the cellular internalization process. Therefore, cellular uptake amounts of curcumin decreased with the augmentation of particle size.

Cytotoxicity of liposomes

The cytotoxicity of liposomes was evaluated to reflect the safety of liposomes. Cytotoxicity of different types of liposomes is shown in Figure 6. Comparing with blank control (cytotoxicity = 100%), little cytotoxicity is observed in PGL and conventional liposomes ($p > 0.05$). From the results, PGL and conventional liposomes had similar and high safety for CHO cell line.

Conclusions

Liposome is one of the most promising microparticle carrier for therapeutic agent. However, compositions in liposomal formulation play very important roles in improving drug cellular uptake[19–24]. In this paper, a new type of flexible liposomes–PGL was prepared and its related properties were evaluated. With propylene glycol used as elasticity modulator and trehalose used as lipid membrane stabilizer, PGL showed more advantages than conventional liposomes. From physical characterization

observation, PGL had smaller particle size, higher drug encapsulation efficiency, better deformability and stability than conventional liposomes. Cellular uptake and cytotoxicity experiments showed that PGL had strong effect on curcumin cellular uptake and low cytotoxicity.

Though the mechanism was not clear, the interaction between PGL compositions and their physical properties played an important role in facilitating cellular uptake. Further study should be carried out to investigate the mechanism from cell level. In addition, a rational designed liposome carrier could selectively deliver the loaded drug to the targeting site, which resulted in maximized therapeutic efficacy and minor adverse effects[25–27]. So future study will focus on PGL biodistribution *in vivo*. Based on the results in this paper, PGL might be used as a promising carrier system for curcumin.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this paper.This research was supported by the National Natural Science Funds (30870755, 81071277, 81071164), Natural Science Foundation of Zhejiang Province (Y2110587, Y2080915), Natural Science Foundation of Beijing (7112100), Medicine and Health Grant from Wenzhou Bureau of Science and Technology (S20100049, H20100017, Y20100029), Zhejiang Province funds for Health Department (2010ZQ007, 2011ZDA017), Zhejiang Province funds for Education Department(Y201016664). The authors (Lu Zhang, Cui-Tao Lu and Wen-Feng Li) contributed equally to this work.

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